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Briegel, Hans ; Rezzonico, Licia

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## CONCENTRATION OF HOST BLOOD PROTEIN DURING FEEDING BY ANOPHELINE MOSQUITOES (DIPTERA: CULICIDAE)<sup>1</sup>

Hans Briegel and Licia Rezzonico<sup>2</sup>

**Abstract.** During blood feeding, *Anopheles stephensi* ingested 2 to 10  $\mu$ l of blood. It took such large meals by releasing large amounts of a red rectal fluid as it fed. Although the fluid contained intact erythrocytes, the female was able to concentrate the dietary protein in the midgut by a factor of about 2. Two larger species, *An. albimanus* and *An. quadrimaculatus*, consuming larger meals, released a clear rectal fluid and also concentrated their blood meals without losing protein. Therefore, gravimetric determinations of blood consumption would be misleading in *Anopheles*. Concentration of blood proteins during the act of feeding, before diuresis, is unique for *Anopheles* and led to increased fecundity when compared to blood meals given by enema. Similar to results with *Aedes*, fecundity was significantly lower with human than with guinea pig blood. It was further demonstrated that female *Anopheles* actually refeed on successive days when a host was offered. In contrast to *Aedes*, subsequent blood meals produced an increase in the number of maturing oocytes.

Despite their important role as primary vectors of plasmodia causing primate malaria, female *Anopheles* have received comparatively little attention from insect physiologists, possibly because aedine and culicine mosquitoes are more easily reared and handled. Aedine and culicine mosquitoes generally require 1 blood meal for each gonotrophic cycle, while anopheline mosquitoes in the laboratory are routinely fed at least twice per gonotrophic cycle, sometimes even daily (Gerberg 1970, Horsfall 1972). To our knowledge, however, the physiological implications of these feeding regimes have never been analyzed.

As in many hematophagous insects, e.g., *Rhodnius* (Maddrell 1964) and *Glossina* (Gee 1975), a conspicuous diuresis begins in mosquitoes shortly after ingestion of blood (Boorman 1960, Stobbart 1977, Jones & Brandt 1981, Williams et al. 1983). Nijhout & Carrow (1978) have investigated diuresis in *Anopheles freeborni* Aitken and ascribed its control to a diuretic hormone. In all these studies the experimental analysis of diuresis began with cessation of blood feeding and continued for roughly 1 h thereafter. In *Anopheles*, however, excretion of

rectal fluid starts a few seconds after piercing the skin of the host and ingestion of blood. A notable occurrence during the feeding of colonies of *An. stephensi* Liston is the excretion of a bright red fluid. For convenience we will call these excretions rectal fluid, because their origin has not been investigated. Excretion of rectal fluid during feeding is arbitrarily defined as prediuresis, and aqueous excretion after feeding has stopped as diuresis.

In this report we will analyze prediuretic excretion quantitatively and compare it among 3 anopheline species of different subgenera and body size. At the same time, the results will be related to fecundity of *Anopheles*, and possible behavioral adaptations will be discussed. It is particularly interesting to compare our results with quantitative data reported for *Ae. aegypti*, the yellow fever mosquito (Briegel 1985).

### MATERIALS AND METHODS

The following species were used for our experiments: *Anopheles (Cellia) stephensi*, *An. (Nyssorhynchus) albimanus* Wiedemann, and *An. (Anopheles) quadrimaculatus* Say. Larvae were reared on a high-protein diet (Lea 1964), with 300 larvae per pan in 450 ml distilled water. The adults eclosed within a 2-day period and were of uniform size. They were kept continuously at 27 °C, 85% RH, under long-day conditions (16L:8D), with sunset and sunrise simulated by continuous dim light for 30 min.

Experimental blood meals were given either in measured volumes by enema (Briegel & Lea 1975) or by feeding the females individually on a restrained guinea pig until repletion, i.e., until withdrawal of the proboscis (generally between 1 and 10 min).

For total nitrogen measurements, the excretory droplets were accumulated on a piece of cover slip held with forceps below the anus; evaporation was considered irrelevant for these determinations. For the hemoglobin determinations, however, a small glass vial containing 500  $\mu$ l of water was held near the anus to catch all rectal fluid released during feeding and to prevent denaturation of the he-

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moglobin. After the experimental blood meals, females were kept individually in reacting tubes ( $10 \times 75$  mm). For the first 24 h they were kept without water; then they were provided with cotton pads soaked in 10% sucrose solution for the rest of the experiment. This combination kept mortality below 5–10%. After 2 or 3 days the ovaries were dissected in saline and the mature oocytes counted; in the experiments on fecundity, ovaries were subsequently used for nitrogen determinations.

In several experiments the volume of the blood meal taken by feeding was estimated by weighing individual females before and after on a microbalance (Cahn DTL). For this purpose the females were anesthetized weakly with  $\text{CO}_2$ ; only those recovering instantly as indicated by avid biting activity were used for the experiments. Some of the females were used for midgut dissection to measure total nitrogen. Dissection was carried out in saline (0.6%), and each midgut was analyzed individually after briefly washing it in a new drop of saline. Since the nitrogen content of the epithelium was below 2% of the blood bolus (unpubl. observ.), it was considered negligible.

Total nitrogen of the samples was determined through Nesslerization of a Kjeldahl digest (Minari & Zilversmit 1963). For the hemoglobin determinations we used a Drabkin reagent (Briegel et al. 1978) calibrated with a standard human hemoglobin (525-18, Sigma Chemical Co., St. Louis, MO). In most experiments the same guinea pig was used as a host. The variations in its blood nitrogen or hemoglobin titers were averaged through periodic sampling by heart puncture, performed under anesthesia.

Linear regression lines for the data were computed using a commercially available program (HP-67).

## RESULTS

### *Blood meal and simultaneous excretion of rectal fluid*

Forty-eight *An. stephensi* females were fed individually to repletion on a restrained guinea pig. The total nitrogen was compared in (1) the pooled droplets of rectal fluid collected during feeding, (2) the blood in the midgut, and (3) a sample of blood from the host. The data and the linear regressions are presented in Fig. 1, where the midgut nitrogen and excretory nitrogen are each plotted against the nitrogen content of the complete

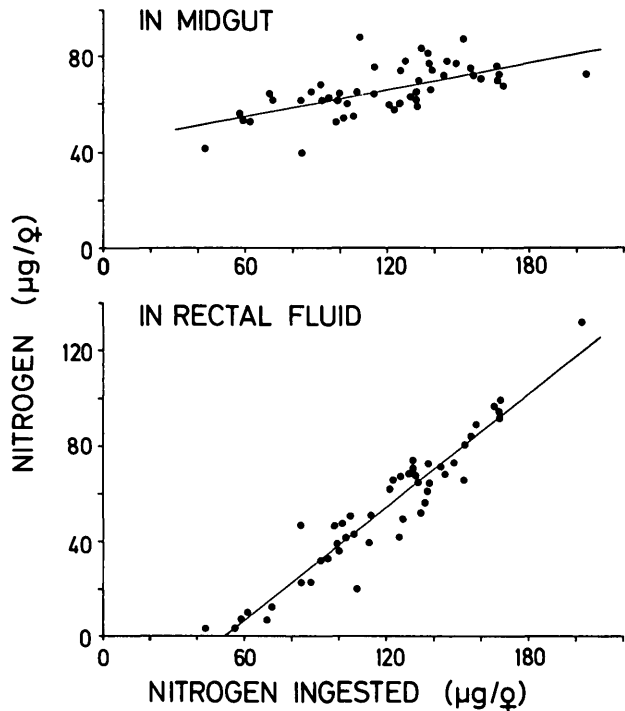


FIG. 1. Nitrogen retained in the midgut of *An. stephensi* or excreted with rectal fluid during feeding to repletion on a restrained guinea pig. Nitrogen ingested is the sum of the measurement from the midgut plus rectal fluid. Each point represents 1♀; linear regressions are given for midgut ( $r = 0.661$ ) and for rectal fluid ( $r = 0.958$ ) from 48♀.

blood meal (i.e., excretory plus intestinal nitrogen). We assumed that no substantial protein was degraded or absorbed during the feeding and collecting period. The maximal amount of intestinal nitrogen was about  $80 \mu\text{g}/\text{q}$  (Fig. 1). This figure further indicates that rectal fluid was not released before 40 to  $60 \mu\text{g}$  of nitrogen had been retained in the midgut. Since the nitrogen concentration of the host blood was  $19.82 \pm 1.44 \mu\text{g}/\mu\text{l}$  ( $n = 5$ ), the maximal sum of nitrogen (about  $200 \mu\text{g}$ ) indicated a blood consumption of up to  $10 \mu\text{l}$ .

In Fig. 2 the midgut nitrogen (upper line) is compared with the volume of blood ingested, as determined by weight gain of these same females (lower line). Generally speaking, all midgut nitrogen measurements were consistently about  $30 \mu\text{g}$  higher than the extrapolation based on the weight increase and the host blood nitrogen (lower line). Thus blood meal nitrogen titers increased during feeding, and weighing the females underestimated the blood consumption substantially.

Total blood consumption per female was calculated by adding the average midgut and excretory nitrogen data and then dividing by the nitro-

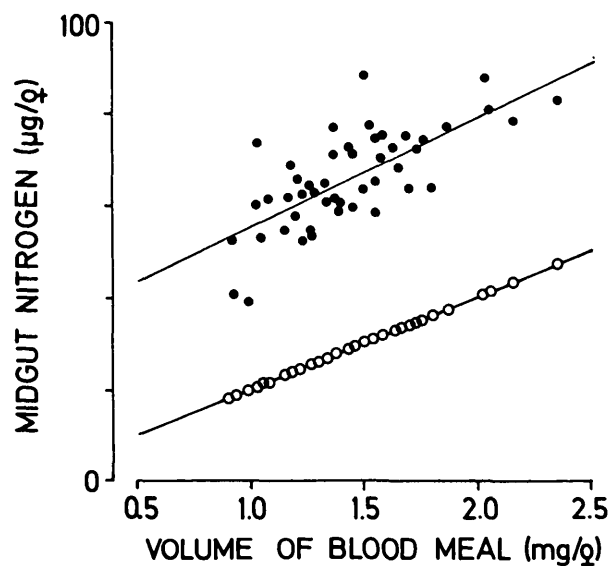


FIG. 2. Midgut nitrogen in *An. stephensi* after feeding to repletion on a guinea pig (●) and the amount of nitrogen ingested as calculated from the weight gains of 48♀ and the nitrogen content in the host blood (○). Each point represents 1♀ and linear regressions are given ( $r = 0.72$  for ●;  $r = 0.96$  for ○).

gen concentration in the host blood (Table 1). This revealed an average blood consumption of  $6 \mu\text{l}/\text{♀}$  *An. stephensi*, with individual variations between 2 and  $10 \mu\text{l}$ .

Dividing the average midgut nitrogen by the weight of the blood (after its conversion to nitrogen) led to the concentration factor of 2.3. The loss of nitrogen through simultaneous excretion of rectal fluid varied tremendously between 2 and  $130 \mu\text{g}/\text{♀}$ ; the average of  $54 \mu\text{g}$  was 45% of the total blood consumption.

When we gave measured amounts of blood by enema to *An. stephensi* in  $0.5\text{-}\mu\text{l}$  increments from 0.5 to  $3 \mu\text{l}$ , we found that blood meals larger than  $2 \mu\text{l}$  could never be injected without rupturing the midgut. Obviously, this quantity was the maximal volume of the distended abdominal midgut for this species reared under our conditions. This volume also coincided with the amount of blood retained in the midgut before the prediuretic excretion of rectal fluid began (see above). Relating this to the range of blood consumption of 2 to  $10 \mu\text{l}$  (Table 1), it is evident that a substantial concentration of the blood protein took place in most females.

Since 80% of the vertebrate blood nitrogen occurs in the hemoglobin (Bursell 1965), and because of the red color of the rectal fluid, this experiment was repeated, this time measuring the hemoglobin in the pooled fluid, the midgut, and host blood samples. Exactly the same result was obtained (Fig. 3). At the end of feeding, the midgut contained an average of  $499.9 \mu\text{g}$  hemoglobin (range 313–676

TABLE 1. Comparison of the blood meal concentration among 3 anopheline species. All values are expressed as microlitres or were converted to micrograms of nitrogen, where applicable. N = nitrogen.\*

	<i>An. stephensi</i> ( <i>n</i> = 48♀)		<i>An. albimanus</i> ( <i>n</i> = 57♀)		<i>An. quadrimaculatus</i> ( <i>n</i> = 44♀)	
	$\mu\text{l}/\text{♀}$	$\mu\text{g N}$	$\mu\text{l}/\text{♀}$	$\mu\text{g N}$	$\mu\text{l}/\text{♀}$	$\mu\text{g N}$
Wt. of blood/♀**						
Mean (A)	1.4	28.5	1.5	29.3	2.1	40.6
Range	0.9–2.4		0.6–2.8		1.3–3.4	
N/midgut						
Mean (B)		65.7		54.7		87.1
Range		38.9–87.1		28.9–81.5		58.9–123.9
N excreted during feeding						
Mean (C)		53.8		1.7***		—
Range		2.3–130.5		1.3–2.8		
Amt. of blood ingested/♀						
Mean (B + C)	6.0	119.5	2.9	56.4	4.4	87.4
Range	2.2–10.2		1.5–4.2		3.0–6.3	
Conc. factor						
Mean (B:A)		2.3		1.9		2.2
Range		1.4–3.1		1.0–2.8		1.5–3.1

\* All ♀♀ were fed on the same guinea pig with a nitrogen content of  $19.82 \pm 1.44 \mu\text{g}/\mu\text{l}$  blood (*n* = 5).

\*\* Increase of ♀ fresh weight assumed to equal volume of blood retained in the midgut.

\*\*\* 9♀.

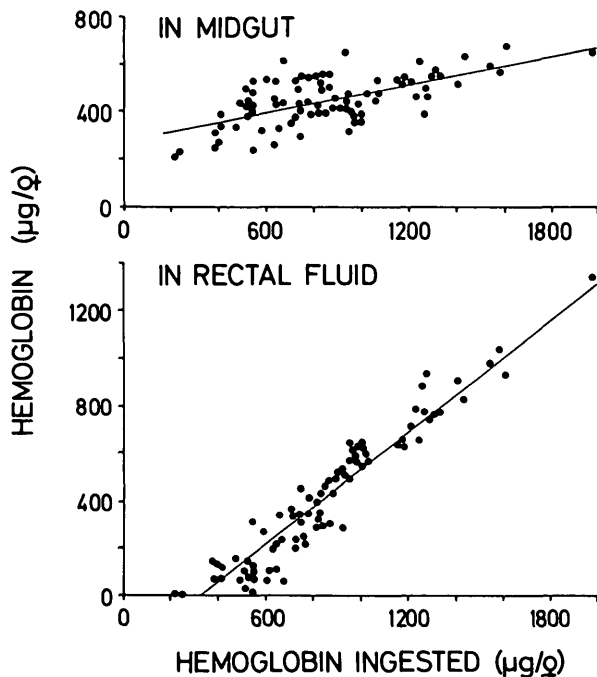


FIG. 3. Hemoglobin retained in the midgut of *An. stephensi* and excreted with rectal fluid during feeding to repletion on a restrained guinea pig. Hemoglobin ingested is the sum of the measurement from midgut plus rectal fluid. Each point represents 1♀; linear regressions are given for midgut ( $r = 0.633$ ) and for rectal fluid ( $r = 0.949$ ) from 83♀.

$\mu\text{g}$ ,  $n = 30$ ), while in the corresponding samples of rectal fluid we detected an average of  $407.5 \mu\text{g}$  (range  $12\text{--}930 \mu\text{g}$ ). Based on the hemoglobin titer in the host blood ( $142.6 \pm 3.5 \mu\text{g}/\mu\text{l}$ ,  $n = 3$ ), the average total hemoglobin ingested was roughly  $6.5 \mu\text{l}$  of guinea pig blood (range  $2.7\text{--}14.0 \mu\text{l}/♀$ ), while only a volume of  $1.4$  to  $2 \mu\text{l}$  was retained in the midgut. When the midgut hemoglobin was compared to the values obtained by weighing the females (Fig. 4), it was ca.  $300 \mu\text{g}$  higher than the extrapolated values. Thus, the error in estimating blood ingestion by gravimetry is comparable to the one noted before with nitrogen determinations.

To prove that the red rectal fluid excreted by *An. stephensi* during feeding represented excess blood, the following experiments were carried out. Giemsa-stained blood smears were prepared from the midgut contents immediately after feeding ended, as well as from the rectal fluid, and both were compared with smears from native host blood. Under the microscope, they all appeared identical, i.e., the rectal fluid contained intact erythrocytes, although at a much smaller density per optical field than the midgut preparation or the host blood (ca. 50%). Furthermore, at the end of feeding, the

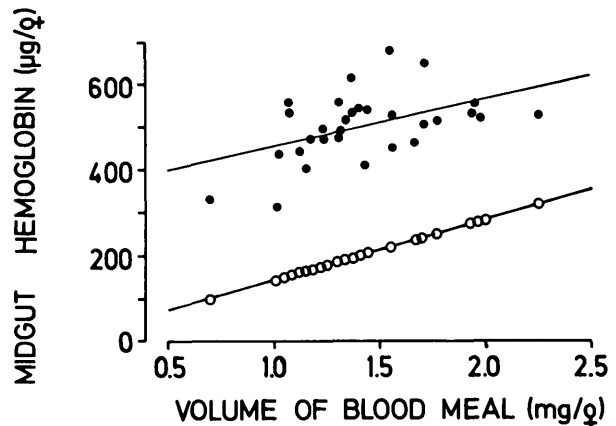


FIG. 4. Midgut hemoglobin in *An. stephensi* after feeding to repletion on a guinea pig (●) and the amount of hemoglobin ingested as calculated from the weight gains of 30♀ and the content in the host blood (○). Each point represents 1♀ and linear regressions are given ( $r = 0.47$  for ●;  $r = 1.00$  for ○).

pooled rectal fluid, the entire midgut contents from the same female, and  $2 \mu\text{l}$  of host blood were each subjected to an acid hydrolysis ( $6 \text{ N HCl}$ ,  $130^\circ\text{C}$ ,  $13 \text{ h}$ ), and the molar distribution of the amino acids was compared by the percentage of their totals. All 3 samples had identical relative compositions, indicating the absence of selective absorption or retention of specific amino acids (or peptides or proteins) from the blood meal during feeding.

#### Comparison with other *Anopheles* species

Blood feeding and simultaneous prediuretic excretion in *An. stephensi* was compared with *An. albimanus* and *An. quadrimaculatus*. Since in both species the rectal fluid is clear, apparently lacking any hemoglobin, we measured only total nitrogen in the samples.

Fifty-seven *An. albimanus* were fed individually on the same guinea pig, and the samples were collected as before. The total nitrogen per midgut at the end of feeding averaged  $54.7 \mu\text{g}$  (Table 1), but only  $1.7 \mu\text{g}/♀$  ( $n = 9$ ) was recovered in the large volume of rectal fluid, corresponding to less than 5% of the midgut content. The actual blood consumption in this species was between  $1.5$  and  $4.2 \mu\text{l}/♀$ , with an average concentration factor of  $1.9$ .

Forty-four *An. quadrimaculatus* fed on the same guinea pig were treated and analyzed in the same way. The total nitrogen per midgut at the end of feeding was  $87 \mu\text{g}$ , yielding an average blood consumption of  $4.4 \mu\text{l}/♀$ ; the concentration factor was  $2.2$  (Table 1). In this experiment the nitrogen excretion was not quantified, but comparing the midgut nitrogen with blood consumption indicated that

TABLE 2. Fecundity of *An. stephensi* after blood meal from guinea pig or man, administered by feeding on the host or by enema (mean  $\pm$  SE,  $n$  = no. determinations).

BLOOD MEAL AND HOST	AMT. OF BLOOD IN MIDGUT (mg/g)	OVARIES		$n$
		NO. EGGS/g	NITROGEN CONTENT ( $\mu$ g/g)	
Feeding on guinea pig*	0.7–1.0	86 $\pm$ 18	13.5 $\pm$ 3.0	8
	1.0–1.5	95 $\pm$ 22	16.8 $\pm$ 3.4	19
	1.5–2.0	122 $\pm$ 13	21.7 $\pm$ 0.9	6
	2.0–2.2	157 $\pm$ 9	26.4 $\pm$ 3.0	3
Enema: guinea pig blood*	1.0	36	10.0	2
	1.5	60 $\pm$ 9	14.2 $\pm$ 1.4	17
	2.0	88 $\pm$ 14	18.6 $\pm$ 1.8	15
Enema: human blood**	1.0	0		6
	1.5	34 $\pm$ 4	8.2 $\pm$ 1.2	11
	2.0	50 $\pm$ 12	11.6 $\pm$ 2.0	13

\* Nitrogen content: 25.7  $\pm$  0.9  $\mu$ g/ $\mu$ l ( $n$  = 5).

\*\* Nitrogen content: 33.4  $\pm$  1.8  $\mu$ g/ $\mu$ l ( $n$  = 5).

nitrogen excreted was less than 1% of the nitrogen ingested (Table 1).

Table 1 further demonstrates that the size range of blood meals ingested was related to the size of the mosquitoes. *Anopheles quadrimaculatus*, the largest species tested, ingested 1.3 to 3.4 mg of blood (50% of all females between 1.6 and 2.2 mg), whereas *An. stephensi*, the smallest species, retained only 0.9 to 2.4 mg blood (50% of all females between 1.2 and 1.6 mg). Conversely, the smallest species showed the highest consumption (2 to 10  $\mu$ l), accompanied by the highest loss of nitrogen through prediuretic excretion, i.e., 2–131  $\mu$ g/g.

#### Significance of blood meal concentration for fecundity

To test the effect of prediuretic blood meal concentration on fecundity, 36 *An. stephensi* were fed to repletion on a guinea pig, while 34 other females were given enemas. In addition, 30 females were given human blood by enema. The number of mature oocytes was counted and compared to the midgut blood content and to the ovarian nitrogen (Table 2).

Fecundity was doubled when blood was taken by feeding as opposed to an enema of guinea pig blood. Enemas of human blood, on the other hand, led to a clearly reduced fecundity, despite its higher nitrogen content. Utilization of the dietary nitrogen was 47 to 51% with enemas of guinea pig blood, but only 16 to 17% with enemas of human blood. To our surprise, the average nitrogen content per egg varied considerably, depending on how the blood meal was acquired: lower after feeding

(guinea pig: 0.17  $\pm$  0.02  $\mu$ g/egg,  $n$  = 36), and significantly higher when given by enema (guinea pig: 0.23  $\pm$  0.03  $\mu$ g/egg,  $n$  = 34; human: 0.24  $\pm$  0.03  $\mu$ g/egg,  $n$  = 24;  $P$  < 0.001 for both hosts).

To our knowledge, the usual practice of feeding *Anopheles* in the laboratory daily never had been subjected to a physiological analysis. Therefore, we tried to clarify whether females having fed once would actually refeed the following day, and whether multiple blood meals were required to complete 1 gonotrophic cycle. Of 25 *An. stephensi* fed to repletion on a guinea pig and thus containing a concentrated blood meal, 17 (68%) refeed 24 h later. In another experiment, the 1st blood meals were given by enema (0.5, 1.0, 1.5, and 2.0  $\mu$ l of guinea pig blood). Twenty-four hours later these females were individually exposed to the guinea pig, and from 50% (initially given 2  $\mu$ l) to 100% (given 0.5  $\mu$ l) refeed readily. Obviously, the lack of blood concentration owing to the enema technique did maintain biting activity.

In a similar experiment with *An. albimanus*, the 1st meals were enemas of 0.5 to 3.0  $\mu$ l guinea pig blood. None of the females receiving 2.5 or 3  $\mu$ l refeed the next day; however, females initially given 0.5 to 1.5  $\mu$ l refeed the following day, and even refeed a 2nd time (i.e., 48 h after the enemas). Each of these blood meals led to an increase in the egg counts. For example, a female given only 0.5  $\mu$ l matured 17 eggs, while after a 2nd meal by feeding, her sister matured 82 eggs; a 3rd meal by feeding produced 128 eggs. In a case with a 1.5- $\mu$ l enema, the 3rd meal produced 185 eggs, in contrast to 52 with the enema alone. Although these results are too scant for statistical analysis, they demonstrate convincingly that fecundity is considerably enhanced by multiple feedings.

#### DISCUSSION

In all 3 malaria vectors tested, the average nitrogen or hemoglobin content of the midgut at the end of feeding was at least double that determined by weighing females. This is the result of prediuretic excretion, i.e., excretion of serum or serum and erythrocytes as blood is ingested. Therefore, protein is accumulated substantially in the anopheline midgut, and the amount of protein available for oogenesis (Table 1, midgut) is comparable to that found in aedine mosquitoes (Briegleb 1985). Since excretion starts during feeding as soon as the midgut is filled, it appears to be an overflow of blood from the distended midgut. The maximal volume that the abdominal midgut could hold

without rupturing was found to be 2  $\mu$ l in *An. stephensi* and 3  $\mu$ l in *An. albimanus*, only  $\frac{1}{2}$  or less of the amount observed in *Ae. aegypti* (6–7  $\mu$ l; Briegel 1985). Regular diuresis, initiated only after termination of feeding in *Aedes* (Boorman 1960, Stobart 1977, Jones & Brandt 1981, Williams et al. 1983) as well as in *Anopheles* (Nijhout & Carrow 1978), serves primarily to reduce the flight weight of the newly fed female. The prediuretic excretion reported here, however, seems to be an additional adaptation evolved by these mosquitoes primarily to compensate for the smaller volume of the midgut and/or its limited elasticity. Indeed, it is followed by normal diuresis as reported in the literature.

There is, however, a considerable difference between *An. stephensi* and the other 2 *Anopheles* tested: *An. albimanus* and *An. quadrimaculatus* have an efficient "filtration system," producing a clear rectal fluid containing little or no nitrogen. This is in contrast to the rectal fluid of *An. stephensi*, which contains erythrocytes. Could this be interpreted as a more primitive evolutionary stage than in other *Anopheles*, perhaps comparable to plant feeding in aphids, which concentrate valuable nutrients by passing excessive fluids?

There are certain disadvantages to this concentrating mechanism. Besides losing protein, the feeding time of *An. stephensi* is prolonged considerably (up to 10 min or more) when compared to the other *Anopheles* species or to *Aedes* (feeding times 1–2 min). A behavioral correlate to this extended feeding might be the biting activity during dusk or night, as is often encountered among anophelines (Horsfall 1972) because reduced defensive behavior of sleeping hosts is likely.

So far it remains an open question as to why *An. stephensi* cannot prevent the loss of protein. One possible explanation might be that the peritrophic membrane is formed much later in this than in other species (Freyvogel & Stäubli 1965).

Accumulation of protein in the midgut through prediuretic excretion is clearly reflected by fecundity: the number of eggs produced per female *Anopheles* after ingesting a "full meal" is similar to or even higher than that in *Ae. aegypti* (Christophers 1960, Briegel 1985). The utilization of the blood meal nitrogen for oogenesis varied between 30 and 40% and was comparable to that in *Ae. aegypti*. Fecundity was equally affected by the source of the blood as it is in *Aedes*: with human blood, fecundity was reduced by  $\frac{1}{2}$  as compared to rodent blood, for the same reasons reported elsewhere

(Briegel 1985). Injecting blood meals by enema, on the other hand, also led to reduced fecundity as compared to feeding, in contrast to *Aedes* where no differences were observed between the 2 routes (Briegel 1985). This is explained by the absence of prediuretic concentration after enemas.

That in both *An. stephensi* and *An. albimanus* most females refed on a host 24 h after a previous blood meal points to 2 other basic differences compared to aedine mosquitoes. First, the humoral inhibition of another blood meal exerted by maturing oocytes (Klowden 1981) seems to be very weak or absent. No other time intervals have been tested yet, but in several cases even a 3rd meal was taken and utilized (see above). Second, as we have indicated before, these subsequent blood meals were utilized for vitellogenesis, which was initiated by the 1st meal. This observation points to different mechanisms regulating fecundity than in *Ae. aegypti*, where oosorption was demonstrated to start about 8 to 12 h after the 1st (small) blood meal (Lea et al. 1978).

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